Characterization of the Sex Steroid Binding Protein of Human Pregnancy Serum. Improvements in the Purification Procedure†

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ABSTRACT: The sex steroid binding protein (SBP) of human pregnancy serum was purified to homogeneity by the sequential use of ammonium sulfate precipitation, affinity chromatography on 5α-dihydrotestosterone-17β-succinyldiaminoethyl-(1,4-butanediol diglycidyl ether)-agarose, and preparative polyacrylamide gel electrophoresis. The yield of pure SBP was improved from 5% as originally reported [Mickelson, K. E., and Petra, P. H. (1975), *Biochemistry 14*, 957] to 34%. Homogeneity of SBP was shown by equilibrium sedimentation ultracentrifugation in 6 M guanidine hydrochloride containing 0.1 M mercaptoethanol which yields a minimum molecular weight of 36 335 ± 525. The protein is also homogeneous when examined by gel electrophoresis in the presence of sodium dodecyl sulfate. A value of 52 000 for the molecular weight is obtained by this method. SBP partially purified from Cohn fraction IV has also a molecular weight of 52 000 by gel electrophoresis in the presence of sodium dodecyl sulfate; that fraction is contaminated with another protein of molecular weight 90 000 which must be removed to obtain homogeneous SBP. The amino acid composition of SBP isolated from pregnancy serum is presented.

The existence of a specific protein (SBP)[1] in the plasma of many species, including humans, which binds androgens and/or estrogens is now well established. Although much has been published on the occurrence of this protein since its discovery in human plasma (Mercier et al., 1965; Rosenbaum et al., 1966), little is known about its physicochemical properties. We have already published a purification method for obtaining homogeneous SBP from human pregnancy serum (Mickelson and Petra, 1975). Now, we report on the physicochemical properties of this protein as well as improvements in its purification.

Materials and Methods

Chemicals. Human pregnancy serum collected at term was obtained from University Hospital, Group Health Cooperative of Puget Sound, and Madigan Army Medical Center. Transferrin (human), hemoglobin (human), ovalbumin, and catalase were obtained from Sigma Chemical Co. Bovine serum albumin (Pentex) was obtained from Miles Laboratories, Inc. Alcohol dehydrogenase (horse liver), alcohol dehydrogenase (yeast), and glucose oxidase (*A. niger*) were obtained from Worthington Biochemical Corp. Carbonic anhydrase and glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle) were obtained from Calbiochem. Sepharose 4B was obtained from Pharmacia Fine Chemicals. All reagents used for polyacrylamide gel electrophoresis were obtained from Canalco Inc. 5α-[1,2-^{3}H]Dihydrotestosterone (44 Ci/mmol) was obtained from New England Nuclear. Radioactive steroids were at least 98% pure. DEAE-cellulose filter-paper disks (Whatman DE-81, 2.3-cm diameter) were obtained from H. Reeve Angel and Co. Ethylenediamine was obtained from Eastman Kodak Co. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide was obtained from either Ott Chemical Co. or Aldrich Chemical Co., Inc. All other chemicals were reagent grade. Cohn fraction IV was a gift from Dr. Duane Schroeder of the Cutter Laboratories, Berkeley, Calif.

Assay of 5α-Dihydrotestosterone Binding Activity. The filter assay was used as previously described (Mickelson and Petra, 1974). The filter disks were placed in scintillation vials and counted 2 h after the addition of scintillant (4 g of Omnifluor/L of toluene).

Ammonium Sulfate Fractionation of Serum. Human pregnancy blood lost during delivery was allowed to clot at 4 °C for 1–3 weeks. The serum was decanted away from the clot and filtered through cotton gauze. The serum was clarified by centrifugation at 16 000*g* for 10 min. Finely divided ammonium sulfate (295 g) was slowly added to each liter of serum. Usually, 4 L of serum was processed at one time. The mixture was stirred at 4 °C for at least 2 h and sometimes up to 18 h before the precipitate was recovered by centrifugation at 16 000*g* for 20 min. The precipitate was washed twice with 2 L of ammonium sulfate solution (295 g/L). The precipitate was dissolved in 500 mL of 10 mM Tris (pH 7.4, 4 °C) and dialyzed against 18 L of distilled water at 4 °C. The external dialysis water was changed twice during the 48-h dialysis procedure. The solution was clarified by centrifugation at 16 000*g* for 10 min. The supernatant was lyophilized and stored at −20 °C.

Synthesis of [1,2-^{3}H]Dihydrotestosterone 17β-Succinate. [^{3}H]DHT (5 mmol, 5μCi/mmol) and 14.5 mmol of succinic anhydride were dissolved in 10 mL of warm, freshly distilled pyridine. The mixture was refluxed until no DHT remained, as determined by TLC ($CHCl_3$–MeOH, 95:5). Completion of the reaction usually took 8–10 h. The mixture was then al-

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[1] Abbreviations used are: SBP, sex steroid binding plasma protein (although this protein has also been called TeBG and SHBG, the accepted nomenclature of SBP was assigned at the 4th meeting of the International Study Group for Steroid Hormones in Rome, Dec. 1969, following the suggestions of Drs. E. E. Baulieu and U. Westphal); DHTS, 5α-dihydrotestosterone 17β-hemisuccinate; $NaDodSO_4$, sodium dodecyl sulfate; CBG, corticosteroid-binding globulin; DEAE, diethylaminoethyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

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lowed to cool to room temperature, which resulted in precipitation of unreacted succinic anhydride. The solution was filtered and the pyridine was evaporated under vacuum. Fifty milliliters of $CHCl_3$ was added and the succinic acid formed was removed by extracting three times with 100 mL of water. The $CHCl_3$ was dried with anhydrous sodium sulfate and the color was removed by charcoal adsorption (1 g, 30 min at 25 °C). The $CHCl_3$ was evaporated. The product was crystallized from acetone by the addition of water. This procedure resulted in a yield of 60–80%. The melting point was 170 °C. Elemental analysis was performed by Schwarzkopf Microanalytical Laboratory (Woodside, N.Y.). Anal. Calcd for $C_{23}H_{34}O_5$: C, 70.72; H, 8.79; O, 20.48. Found: C, 70.75; H, 8.86; O, 20.73.

Synthesis of Diaminoethyloxirane–Agarose. The method used is essentially that described by Sundberg and Porath (1974). One hundred milliliters of Sepharose 4B was added to 33 mL of 1,4-butanediol diglycidyl ether plus 33 mL of 0.6 M NaOH containing 66 mg of sodium borohydride. The mixture was gently agitated for 15 h at 25 °C by a water-bath shaker. The oxirane–agarose was washed with 4 L of water and then gently suspended in 100 mL of water containing 15 mL of ethylenediamine (pH 10) for 16 h at 25 °C. The agarose derivative was then washed with 2 L of water, 2 L of borate buffer (0.1 M, pH 8.0), 2 L of acetate buffer (0.1 M, pH 4.0), and 2 L of water. Incorporation of amino groups was detected by reaction of the derivatized agarose with trinitrobenzenesulfonate (Cuatrecasas, 1970).

Coupling of Steroids to Aminoalkyl Derivatives of Agarose. Diaminoethyloxirane-substituted agarose gel was equilibrated with 70% dioxane. One gram of dihydrotestosterone 17β-succinate was dissolved in 100 mL of 70% dioxane and added to 100 mL of the diaminoethyloxirane-substituted agarose. The pH was adjusted to 5 and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (5 g) was added to the mixture in two equal portions 4 h apart. The mixture was gently agitated on a mechanical shaker for 15 h at 25 °C. The substituted gels were then washed at room temperature on a Buchner funnel without suction with 10 L of 80% methanol, 10 L of 50% methanol, and 5 L of water. Washing was performed slowly and over a 1–2-week period.

Procedure for Affinity Chromatography. Approximately 10 g of lyophilized powder obtained by ammonium sulfate precipitation of human pregnancy serum was dissolved in 200 mL of 10 mM Tris containing 0.5 M NaCl (pH 7.4, 4 °C). The protein solution was gently stirred for 5 h with 50 mL of affinity adsorbent diluted fivefold with unsubstituted Sepharose 4B. The mixture was poured into a column (5-cm diameter) and washed with 10 mM Tris containing 1 M NaCl until the absorbance at 280 nm was negligible. The agarose was then removed from the column and stirred for 8–12 h at 4 °C with 300 mL of 10 mM Tris containing 10^{-5} M [^{3}H]DHT (0.04 Ci/mmol), 0.5 M NaCl, and 10% glycerol. The mixture was then placed in a Buchner funnel (coarse) and washed with 100 mL of the above buffer at 4 °C. The eluted protein was concentrated to 5–10 mL and dialyzed against buffer [10 mM Tris, 0.1 M NaCl, 10^{-5} M [^{3}H]DHT (0.04 Ci/mmol), 10% glycerol] using an Amicon Model TCF-10 thin-channel system with PM-30 membranes. In our more recent work we have changed to PM-10 membranes after discovering that SBP passes across PM-30 membranes.

Preparative Polyacrylamide Gel Electrophoresis. Preparative gel electrophoresis was performed with a Canalco "Prep Disc" apparatus according to their instruction manual. Polyacrylamide gels (5%) were prepared by the method described by Davis (1964) as modified by Shuster (1971). Polymerization was performed at room temperature and the gels were allowed to remain at room temperature for 4 h before bringing to 4 °C. A 1-mL solution containing up to 30 mg of protein was fractionated in a gel having a diameter of 2.5 cm and length of 3 cm. Electrophoresis was performed at 20 mA (400–500 V) at 4 °C and the elution chamber was eluted at a rate of 60 mL/h with 0.375 M Tris-HCl, pH 8.9. The active fractions were pooled, concentrated to 3–5 mL, dialyzed against 10 mM Tris, 0.1 M NaCl, DHT (approximately 10^{-5} M), and 10% glycerol, and stored at 4 °C.

Sodium Dodecyl Sulfate Gel Electrophoresis. Analytical gel electrophoresis in the presence of $NaDodSO_4$ was performed according to the method described by Weber et al. (1972). Protein samples were denatured in 1% $NaDodSO_4$ and 1% 2-mercaptoethanol at 100 °C for 5 min. Samples (50 μL) containing usually 20 μg of protein were applied per gel.

Equilibrium Sedimentation Ultracentrifugation. Ultracentrifugation was performed in a Spinco Model E ultracentrifuge using a six-channel Yphantis (1964) centerpiece. Kodak II-G photographic plates were used to record the Rayleigh interference patterns. The data were analyzed on a Nikon 6C microcomparator controlled by a PDP-12 computer (DeRosier et al., 1972). Baseline data were obtained at the end of a run by vigorous shaking of the cells and recentrifuging at low speed (Horbett and Teller, 1972). Lyophilized protein was weighed out and dissolved in 6 M guanidine hydrochloride containing 0.1 M mercaptoethanol. The protein solution (1 mL, 0.6 mg/mL) was dialyzed for at least 48 h with 10 mL of the above solution at 25 °C. The appropriate sample concentrations (0.6, 0.4, and 0.2 mg/mL) were obtained by dilution with the dialysate. Molecular weights were calculated by computer by the method of Teller (1973).

Apparent Specific Volume. The apparent specific volume of SBP was calculated from the relative amount of amino acid residues, as determined by amino acid analysis and as described by Cohn and Edsall (1943) using the data of Longsworth (1953). The apparent specific volume in guanidine hydrochloride was determined to be 0.707 mL/g by the method of Lee and Timasheff (1974).

Determination of Amino Acid Composition. Pure protein (50–100 μg) was hydrolyzed in 0.5 mL of 6 N HCl at 110 °C for 24, 48, and 96 h in evacuated tubes that had been flushed with nitrogen. Cystine and cysteine were determined as cysteic acid after performic acid oxidation by the method of Moore (1963). Isoleucine and valine values were calculated from the 96-h hydrolysis. Analyses were determined using a Durrum Model D-500 amino acid analyzer equipped with an electronic integrator. Tryptophan content was determined by the method of Edelhoch (1967) after dissolving lyophilized SBP (200 μg) in 6 M guanidine hydrochloride (1 mL).

Carbohydrate Analysis. Standard colorimetric methods (Winzler, 1955) were employed for hexose, *N*-acetylhexosamine, and fucose. Sialic acid was determined by the method described by Warren (1959). Because of limited quantities of pure protein, reaction volumes were scaled down by a factor of 10 so that 100 μg (dry weight of lyophilized protein) of protein sufficed for each analysis utilizing micro spectrophotometer cells. Results are expressed as % carbohydrate/weight of protein.

Protein Determination. Protein concentration was determined by the method of Lowry et al. (1951). Bovine serum albumin (Pentex) was used as the standard. Solutions were read at 650 or 750 nm (for lower protein concentrations) in a Beckman DB-GT spectrophotometer.

Determination of Radioactivity. Aqueous samples of filter paper disks were added to scintillation vials containing 4–10

TABLE I: Human SBP Purification Scheme.

Step	mg of protein	ng of DHT bound[a]	Sp act.[b]	Cumulative purif.	Cumulative yield, %
Serum (1 L)	42 300	53 500	1.26	1	100
$(NH_4)_2SO_4$ ppt	8 492	32 100	3.78	3	60
Affinity chromatography	29.3	25 680	876	695	48
Prep PAGE[c]	3.40	18 190	5350	4246	34

[a] The filter assay method was used to measure specific DHT-binding activity. [b] Specific activity is expressed as ng of DHT bound/mg of protein. [c] Preparative polyacrylamide gel electrophoresis.

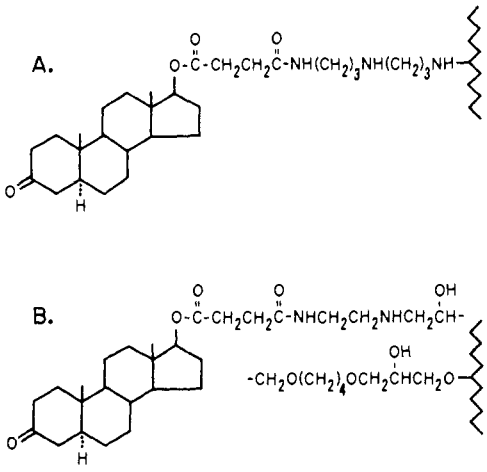

FIGURE 1: Dihydrotestosterone derivatives of agarose used in the purification of SBP. (A) 5α-Dihydrotestosterone-17β-succinyl-(3,3-diaminodipropylamine)-agarose. (B) 5α-Dihydrotestosterone-17β-succinyl-diaminoethyl-(1,4-butanediol diglycidyl ether)-agarose.

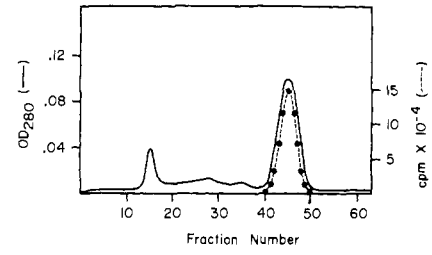

FIGURE 2: Preparative polyacrylamide gel electrophoresis of partially purified human SBP (6 mg) obtained by affinity chromatography. The 5% acrylamide gel measured 2.5 (diameter) × 3.0 cm (height). Electrophoresis was performed at 4 °C at 20 mA. Elution was performed at 60 mL/h. Fractions (6 mL) were collected and 30-μL aliquots were removed and assayed by the filter assay method after diluting 1:100 with 10 mM Tris buffer (pH 7.4).

mL of scintillant (4 g of Omnifluor/L of toluene). Essentially, 100% of the radioactivity was extracted from the filters or the aqueous phase, since steroids are more soluble in toluene.

Results

Determination of the Equilibrium Constant of Association (K_a) of DHTS to SBP. We have previously reported the K_a for DHTS to be 6.6×10^7 M^{-1} (Mickelson and Petra, 1975). We have, however, discovered by thin-layer chromatography that the commercial preparation of DHTS (Steraloids, Inc.) was contaminated with DHT, thereby making that previously reported value of K_a for DHTS incorrect as calculated by the "competitive" Scatchard method (Mickelson and Petra, 1975). Using purified DHTS (melting point 170 °C), we recalculated the K_a to be 4×10^6 M^{-1} using the same procedures.

The results indicate that the incorporation of the succinate group at the 17 position of DHT reduces the binding affinity relative to DHT by a factor of 595 instead of 50 as previously reported. However, the binding to SBP is still high enough for the derivative to be used as a ligand for the purification of SBP by affinity chromatography, since the concentration of steroid covalently bound to the agarose is approximately 2 μmol/mL of gel.

Affinity Chromatography of Human SBP. In our previous experiments, diaminodipropylamine served as the spacer to separate the ligand from the agarose matrix (Figure 1A). This adsorbent was initially used to purify human SBP (Mickelson and Petra, 1975). However, it was observed that the steroid continually "leaked" from the column and could only be used once. Tesser et al. (1974) have reported that this is probably due to the instability of the isourea bond formed between the matrix and spacer when linkage is performed by the cyanogen bromide activation method. To increase the stability of the spacer–matrix linkage, a new agarose activation procedure described by Sundberg and Porath (1974) was utilized. A relatively stable ether bond is formed using a bisoxirane (1,4-butanediol diglycidyl ether). This column (Figure 1B) has been used five times over a period of 4 months with very little loss of efficiency in purifying SBP. Elution of SBP from the affinity column was performed at 4 °C instead of 25 °C as previously described (Mickelson and Petra, 1975). This results in a higher yield of active SBP for the affinity chromatography step: 80% (Table I) instead of 21% as previously reported (Mickelson and Petra, 1975). The total yield of protein obtained at 4 °C is increased by 33%, but the specific activity is three times higher which indicates that SBP is destroyed when the elution process is carried out at 25 °C.

Preparative Polyacrylamide Gel Electrophoresis of SBP Isolated from Pregnancy Serum. The Buchler "Poly-Prep" apparatus originally used was replaced by a Canalco "Prep-Disc" apparatus. The Canalco apparatus has the advantage of having a smaller cross-sectional area; thus, smaller quantities of protein may be applied with minimal loss due to dilution. Moreover, the electrophoresis time is reduced, since a lower percent acrylamide gel (5%) may be used with the Canalco apparatus. Figure 2 shows a typical elution pattern. Yields from this step in the procedure are typically 70–80% (Table I). Analytical gel electrophoresis in $NaDodSO_4$ showed the presence of a single polypeptide chain (Figure 3).

Purification of SBP from Cohn Fraction IV. The purification of SBP from nonpregnancy serum was undertaken in order to determine if SBP had the same physicochemical properties when isolated from either pregnancy or nonpregnancy serum. Cohn fraction IV was initially fractionated by ammonium sulfate precipitation by the same procedure used for the pregnancy serum. Next, a DEAE-cellulose chromatography step was included to remove albumin. This consisted

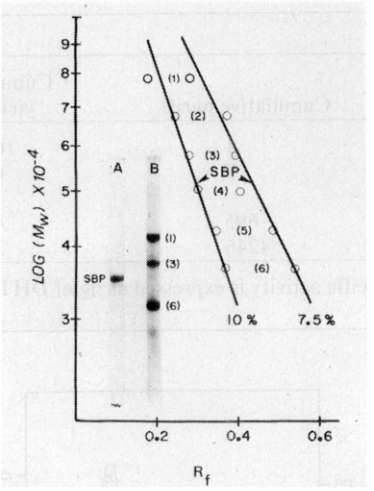

FIGURE 3: Determination of the molecular weight of SBP by $NaDodSO_4$ gel electrophoresis. The plot represents the mobility of standard proteins in 7.5 and 10% gels. The numbers correspond to the following proteins: (1) human transferrin (78 000), (2) bovine serum albumin (68 000), (3) catalase (58 000), (4) H chain of γ-globulin (50 000), (5) ovalbumin (43 000), and (6) alcohol dehydrogenase (37 000). The insert represents 7.5% $NaDodSO_4$ gels of pure SBP (gel A) with standards 1, 3, and 6 (gel B). The dye front migrated 11.5 cm into gels. The gels were cut for photography.

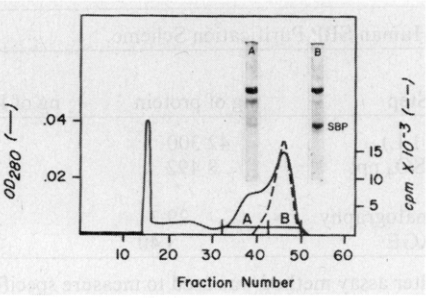

FIGURE 4: Preparative polyacrylamide gel electrophoresis of partially purified human SBP (2 mg) obtained by affinity chromatography of Cohn fraction IV of human plasma. The 5% acrylamide gel measured 2.5 (diameter) × 3.0 cm (length). Electrophoresis was performed at 4 °C at 20 mA. Elution was performed at 72 mL/h. Fractions (7.2 mL) were collected and 50-μL aliquots were removed and assayed by the filter assay method after diluting 1:10 with 10 mM Tris buffer (pH 7.4). $NaDodSO_4$ gels: gel A represents pool A; gel B represents pool B. The R_f of SBP in the analytical $NaDodSO_4$ gels is 0.45 (as in Figure 3). The gels were cut for photography.

of dialyzing the protein fraction against 0.2 M sodium phosphate (pH 6.0) and passing through a column of DE-52 equilibrated with the same buffer. The albumin is bound while the SBP activity passes through the column. This partially purified SBP fraction is then purified by affinity chromatography and preparative polyacrylamide gel electrophoresis as described above. Figure 4 shows the elution profile of the preparative electrophoresis step. Two partially resolved proteins were obtained. $NaDodSO_4$–polyacrylamide gel electrophoresis indicates that the protein fraction possessing the SBP activity (fraction B) contains as a major component the 52 000 molecular weight species observed in pregnancy serum. The specific activity (2400 ng of DHT bound/mg of protein) of this protein fraction indicates that 45% of the protein is SBP based on a comparison of the specific activity of pure SBP isolated from pregnancy serum (5350 ng of DHT bound/ng of protein, Table I). Fraction A of Figure 4 is composed mainly of a protein of molecular weight around 90 000 as determined by $NaDodSO_4$–polyacrylamide gel electrophoresis (gel A, Figure 4). The nature of this protein is presently unknown but is certainly different from SBP, since it does not bind DHT as shown in the electrophoretic pattern of Figure 4.

Characterization of Human SBP

Molecular Weight Determination. (1) Gel Filtration. The molecular weight of human SBP was estimated by gel filtration on agarose–Bio-Gel A, 0.5 m. The logarithm of the molecular weight was plotted against K_{av}, the partition coefficient between the liquid phase and the gel phase which is independent of the compaction of the gel bed (Sephadex: Gel Filtration in Theory and Practice, Pharmacia Fine Chemicals instruction manual). The purified human SBP elutes with an apparent molecular weight of 120 000. When pregnancy serum or normal pooled serum fractionated by ethanol (Cohn fraction IV) is chromatographed on this column, SBP also elutes in the same volume. Thus, the purification procedures do not alter the apparent molecular size of SBP, as determined by this procedure.

(2) $NaDodSO_4$ Gel Electrophoresis. The molecular weight is estimated to be 51 800 ± 700 by $NaDodSO_4$ gel electrophoresis in 7.5 and 10% gels (Figure 3). $NaDodSO_4$ gel electrophoresis in the presence or absence of mercaptoethanol reveals only one band; thus, no subunit structure can be detected by this method. Since $NaDodSO_4$ is known to disrupt noncovalent protein–protein interaction, the possibility exists that human SBP could be a dimer or consist of subunits with the same molecular weight of 52 000. To investigate this, an attempt was made to cross-link subunits by amidination using the bifunctional reagent dimethyl suberimidate (Davies and Stark, 1970). No cross-linkage occurred under the conditions used, strongly suggesting that SBP does not exist as an aggregate of identical subunits.

(3) Equilibrium Sedimentation Ultracentrifugation. Sedimentation equilibrium of human SBP at 5 °C in 10 mM Tris containing 0.10 M NaCl indicated that the protein was heterogeneous at the concentrations tested. Plots of log c vs. r^2 indicated that the system became more heterogeneous with increasing protein concentration, suggesting that the protein was aggregating during centrifugation. The protein samples from the centrifugation procedure were analyzed by $NaDodSO_4$ gel electrophoresis. In the presence of mercaptoethanol, only one band was obtained before and after the centrifugation run. However, in the absence of mercaptoethanol, multiple bands of higher molecular weight species were detected in addition to the major band of 52 000 after centrifugation. Thus, new protein species were formed during centrifugation as a result of mixed disulfide formation. M_n varied from 60 000 to 100 000 in this heterogeneous system. The molecular weight of SBP was therefore estimated by sedimentation equilibrium in 6 M guanidine hydrochloride containing 0.1 M mercaptoethanol. The results shown in Figure 5 indicate that the protein is homogeneous under these conditions. The minimal molecular weight calculated from these data by the method of Teller (1973) is 36 335 ± 525 (Figure 5).

Amino Acid Composition and Partial Specific Volume. Human SBP was found to have a total carbohydrate content of 18%. The molecular weight of the polypeptide backbone of SBP was therefore calculated to be 29 795 by taking 82% of the minimal molecular weight 36 335. The value of 29 795 was used to calculate the amino acid composition shown in Table II. The analysis represents the average of seven different preparations, while the carbohydrate content was performed on a pool of two preparations.

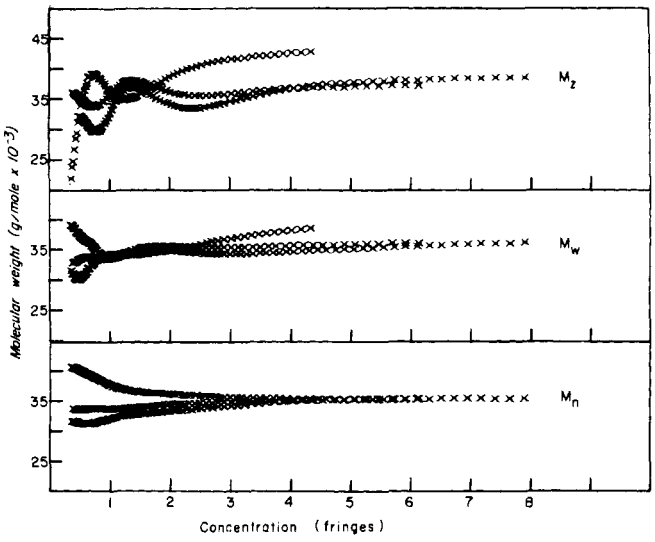

FIGURE 5: Molecular weight distribution of human SBP obtained by equilibrium sedimentation. Data presented are computer-generated plots of molecular weight vs. concentration (fringes) from analysis of a Rayleigh plate. Initial loading concentrations of approximately 0.6, 0.4, and 0.2 mg/mL were centrifuged for 49 h at 26 000 rpm and 23 °C in 6 M guanidine hydrochloride containing 0.1 M mercaptoethanol. M_1 = 36 335 ± 525; M_n = 36 884 ± 1118; M_w = 37 667 ± 138; M_z = 39 509 ± 1149. M_1 represents the smallest molecular weight species calculated according to Teller (1973). M_n, M_w, M_z represents the number-average molecular weight, the weight-average molecular weight, and the z-average molecular weight, respectively. Apparent specific volume (in 6 M guanidine hydrochloride): 0.707 mL/g; ± represents SD.

TABLE II: Amino Acid Composition of Human SBP.

Amino acid	mol/mol of SBP[a]
Cysteic acid	6.0
Aspartic acid	25.1
Threonine	17.7
Serine	27.9
Glutamic acid	25.1
Proline	21.4
Glycine	33.1
Alanine	18.5
Valine	14.3
Methionine	3.1
Isoleucine	8.5
Leucine	35.3
Tyrosine	3.3
Phenylalanine	9.6
Histidine	8.0
Tryptophan	2.4
Lysine	11.3
Arginine	11.6
	% CHO
Hexose	5.0
N-Acetylhexosamine	6.0
Sialic acid	6.5
Fucose	0.5
Sum	18.0

[a] Calculated using a polypeptide molecular weight of 29 795.

Discussion

The modified procedure for the purification of human SBP described in this communication is a marked improvement on that previously reported (Mickelson and Petra, 1975). There are three important changes. First, a new agarose activation procedure (Sundberg and Porath, 1974) resulting in the formation of a stable ether bond between the spacer containing the steroid and the agarose matrix was used to prepare the specific adsorbent. This modification results in a significantly more stable affinity adsorbent which can be reused at least five times after washing each time with organic solvent, as described under Materials and Methods. Second, the temperature for the elution of SBP from the affinity adsorbent has been changed from 25 to 4 °C. Lower temperature improves the yield of SBP at this step from 21%, as previously reported, to 80%. Third, the use of Canalco instrumentation for the preparative electrophoresis step increases the yield from 40 to 70–80%. These three modifications result in an increased overall yield from 5 to 34%. The pure SBP has approximately the same specific activity previously reported (Mickelson and Petra, 1975). The major loss occurs at the $(NH_4)_2SO_4$ step; although at present we have not found a more practical way of accumulating large amounts of the β-globulin fraction containing SBP, we are investigating a batchwise DEAE-cellulose step and the method appears promising. The SBP lost at the affinity chromatography step can be recovered by dialyzing and lyophilizing the eluent. The freeze-dried protein fractions arising from several preparations are then pooled and subjected once more to affinity chromatography. The new procedure allows the preparation of 4–6 mg of pure SBP in approximately 5 days starting from the β-globulin fraction. The modifications have permitted physical characterization of the protein described here and are enabling us to pursue our studies on the chemistry of the binding site. A monospecific antibody has already been prepared (Bordin, Lewis, and Petra, to be published), and further studies on the structural and functional properties of human SBP will be reported later.

Three different values for the molecular weight of human SBP can be obtained, depending upon the method of analysis. SBP in serum, as well as in the pure state, elutes with an apparent molecular weight of 120 000 by gel filtration. An apparent molecular weight of 52 000 is estimated by $NaDodSO_4$–polyacrylamide gel electrophoresis, and equilibrium sedimentation in 6 M guanidine hydrochloride in the presence of 0.1 M mercaptoethanol yields a minimal molecular weight of 36 335 ± 525. The discrepancy in the values can be explained by the presence of high amounts of carbohydrate (18%). Abnormally high apparent molecular weights have been found for other glycoproteins when estimated by gel filtration or $NaDodSO_4$–polyacrylamide gel electrophoresis. For example, the vitamin B_{12} binding protein (33% carbohydrate content) has a molecular weight of 59 300 by sedimentation equilibrium but has an apparent molecular weight of 120 000 by gel filtration (Stenman, 1974). Bovine factor VII (proconvertin), a plasma protein (13% carbohydrate content) which participates in blood coagulation, has a molecular weight of 54 000 by $NaDodSO_4$–polyacrylamide gel electrophoresis and 45 500 by equilibrium sedimentation (Kisiel and Davie, 1975). Also, rabbit CBG has an apparent molecular weight of 48 000 estimated by $NaDodSO_4$–polyacrylamide gel electrophoresis and a molecular weight of 35 000 determined by sedimentation equilibrium (Chader et al., 1972). Segrest et al. (1972) have reported that glycoproteins bind less $NaDodSO_4$ per unit of mass than polypeptide proteins resulting in a lower charge to mass ratio for glycoproteins. This would result in a slower migration of glycoproteins during $NaDodSO_4$–polyacrylamide gel electrophoresis, yielding higher apparent molecular weights. Furthermore, Andrews (1966) has proposed that glycoproteins deviate from the normal gel-filtration behavior of proteins because glycoproteins are presumably more hydrated and thus have an expanded structure which would elute as a higher molecular weight protein. Since the molecular

weights calculated from sedimentation equilibrium data are corrected for the carbohydrate contents by correction of the apparent specific volume, the values obtained by this method are the most reliable. It can be calculated that the change in molecular weight due to a 5% error in the apparent specific volume of the carbohydrate results is only 3%.

Although the various disagreements in the literature concerning the value for the molecular weight of SBP can probably be explained by the presence of the carbohydrate moiety, there is one confusing report which needs to be discussed. Rosner and Smith (1975) have isolated a protein from Cohn fraction IV (alcohol precipitate of outdated pooled plasma) which they identify as SBP. They report an apparent molecular weight of 94 000 by $NaDodSO_4$-polyacrylamide gel electrophoresis. Although direct comparison of their data with our Figure 3 is not possible since no $NaDodSO_4$ gel patterns appeared in their publication, their interpretation, nevertheless, does not agree with our value of 52 000 by this method. Since they report a 32.1% carbohydrate content in comparison to our 18%, the possibility exists that the higher carbohydrate content may bring about the apparently high molecular weight, thereby supporting the view that both proteins differ only in their carbohydrate content. However, the amino acid composition they report is significantly different from ours. Consequently, we are left with the interesting possibility that two different species of SBP may exist in humans.

In order to test this hypothesis, we also purified SBP from Cohn fraction IV using the procedure described in this paper. Our results, shown in Figure 4, indicated that the purified SBP from Cohn fraction IV is contaminated with an impurity which when examined by $NaDodSO_4$-polyacrylamide gel electrophoresis has a molecular weight of about 90 000 (Figure 4, gel A). Since this impurity survives the affinity chromatography step, it may either be nonspecifically adsorbed to the steroid–agarose matrix or some remained on the column as a result of insufficient washing. However, it does not bind DHT as shown in Figure 4 (fraction A). On the other hand, fraction B (Figure 4) containing the radioactivity shows a new band migrating at 52 000 as shown by $NaDodSO_4$-polyacrylamide gel electrophoresis which can only be SBP according to the results of Figure 2. Although we still do not know the nature of the impurity, it seems likely that it is not transferrin, since this latter protein has a molecular weight of 78 000 (Hovanessiam and Awdeh, 1976). Therefore, in conclusion, SBP isolated from either Cohn fraction IV or from pregnancy serum exhibits the same apparent molecular weight of 52 000 by $NaDodSO_4$-polyacrylamide gel electrophoresis. In the case of Cohn fraction IV, care must be taken to remove an impurity having a molecular weight of about 90 000 in order to chemically characterize SBP isolated from pooled plasma.

A value of 36 335 for the molecular weight of SBP indicates that 1 mg of pure protein should bind 7.98 μg of DHT. Table I, however, reveals that only 5.35 μg of DHT is bound under saturation conditions with respect to steroid, or 0.7 mol of steroid/mol of protein. This suggests that about 30% of the SBP molecules in the preparation are inactive. These results are not unexpected, since it is known that SBP is unstable in the absence of steroid and such conditions do exist particularly in the early steps of the purification procedure. If, on the other hand, we assume that all the molecules are active and, in addition, they exist as dimers in the native state composed of two identical subunits (since $NaDodSO_4$ gels yield one band and the monomer is homogeneous in the ultracentrifuge) with a molecular weight of 72 670, the calculated molar ratio is 1.34:1 using the data in Table I. The same calculation assuming a trimer yields a 2.01:1 ratio. Although stoichiometry is achieved in the latter case, one would have to postulate two steroids bound to a complex composed of three polypeptide chains. Therefore, we conclude that, as stated above, 30% of the molecules of SBP are inactive in the final preparation and, although more experiments are needed to establish the quaternary native structure of human SBP, the monomer model is still the most plausible at this time. Along these lines, it is interesting that bovine SBP has recently been found to have an apparent molecular weight of 85 500 in the *native* state with 0.8 binding site/mole (Suzuki et al., 1977). The native protein is composed of three subunits; two of them appear to have a similar molecular weight while the other is slightly larger.

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References

Chader, G. J., Rust, N., Burton, R. M., and Westphal, U. (1972), *J. Biol. Chem. 247,* 6581.

Cohn, E. J. and Edsall, J. T. (1943), Proteins, Amino Acids and Peptides, New York, N.Y., p 370.

Corvol, P. L., Chrambach, A., Rodbard, D., and Bardin, C. W. (1971), *J. Biol. Chem. 246,* 3435.

Cuatrecasas, P. (1970), *J. Biol. Chem. 245,* 3059.

Davies, G. E., and Stark, G. R. (1970), *Proc. Natl. Acad. Sci., U.S.A. 66,* 651.

Davis, B. J. (1964), *Ann. N.Y. Acad. Sci. 121,* 404.

DeRosier, D. J., Munk, P., and Cox, D. J. (1972), *Anal. Biochem. 50,* 139.

Edelhoch, H. (1967), *Biochemistry 6,* 1948.

Horbett, T. A., and Teller, D. C. (1972), *Anal. Biochem. 45,* 86.

Hovanessiam, A. G., and Awdeh, Z. L. (1976), *Eur. J. Biochem. 68,* 333.

Kisiel, W., and Davie, E. W. (1975), *Biochemistry 14,* 4928.

Lee, J. C., and Timasheff, S. N. (1974), *Arch. Biochem. Biophys. 165,* 268.

Longsworth, L. G. (1953), *J. Am. Chem. Soc. 75,* 5705.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Mercier, C., Alfsen, A., and Baulieu, E. E. (1965), *Symp. Steroid Horm., [Proc.], 2nd, 1965,* Ghent.

Mickelson, K. E., and Petra, P. H. (1974), *FEBS Lett. 44,* 34.

Mickelson, K. E., and Petra, P. H. (1975), *Biochemistry 14,* 957.

Moore, S. (1963), *J. Biol. Chem. 238,* 235.

Rosenbaum, W., Christy, N. P., and Kelly, W. G. (1966), *J. Clin. Endocrinol. Metab. 26,* 1399.

Rosner, W., and Smith, R. N. (1975), *Biochemistry 14,* 4813.

Shuster, L. (1971), *Methods Enzymol. 22,* 412.

Stenman, V. (1974), *Biochim. Biophys. Acta 342,* 173.

Sundberg, L., and Porath, J. (1974), *J. Chromatogr. 90,* 87.
Suzuki, Y., Itagaki, E., Mori, H., and Hosoya, T. (1977), *J. Biochem. (Tokyo) 81,* 1721.
Teller, D. C. (1973), *Methods Enzymol. 27D,* 346.
Tesser, G. I., Fisch, H. V., and Schwyzer, R. (1974), *Helv. Chim. Acta 57,* 1718.
Warren, L. (1959), *J. Biol. Chem. 234,* 1971.
Weber, K., Pringle, R. T., and Osborn, M. (1972), *Methods Enzymol. 36,* 3.
Winzler, R. J. (1955), *Methods Biochem. Anal. 2,* 279.
Yphantis, D. A. (1964), *Biochemistry 3,* 297.

Structure of the Oligosaccharides of Three Glycopeptides from Calf Thymocyte Plasma Membranes†

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ABSTRACT: The carbohydrate composition and oligosaccharide structure of three glycopeptides isolated from delipidated calf thymocyte plasma membranes following Pronase digestion have been determined. Five major glycopeptide fractions were separated using Bio-Gel P-6 gel filtration and diethylaminoethylcellulose chromatography. The structure of the oligosaccharide chains of three of these glycopeptides was determined by a combination of sequential degradation with glycosidases and methylation analysis. These oligosaccharide structures consist of complex, highly branched N-linked chains containing at their nonreducing termini the unusual sequence Gal(β1→3)Gal(β1→4)GlcNAc→ as well as the more usual sequence SA(α2→3)Gal(β1→4)GlcNAc→. In addition, one glycopeptide also contains short O-linked chains with the structure Gal(β1→3)GalNAc→Ser(Thr) which have receptor activity for the lectin from the mushroom *Agaricus bisporus.*

Exposed on the outer surface of animal cells are numerous oligosaccharide chains which are constituents of the cells' plasma membrane glycoproteins. These carbohydrate structures have been postulated to be involved in a number of physiologically important functions such as cell-cell interaction, adhesion of cells to the substratum, and migration of cells to particular organs (e.g., in the "homing" of lymphocytes to the spleen and metastasis of tumor cells to preferred sites). If, in fact, cell-surface oligosaccharides serve as the recognition sites for such interactions, it becomes important to know the detailed structure of these carbohydrate moieties. Membrane glycoproteins have been isolated and the structure of the oligosaccharide chains has been determined in only a few cases. The best studied has been the major sialoglycoprotein of the human erythrocyte membrane which has been shown to contain both N-glycosidically linked complex oligosaccharide chains and O-glycosidically linked oligosaccharide chains whose structures have been determined (Thomas and Winzler, 1969, 1971; Kornfeld and Kornfeld, 1971).

In the study described here, another cell type—the thymus lymphocyte—has been examined to determine how many different oligosaccharides are present in the membrane glycoproteins and what their structures are. In previous work (Kornfeld and Siemers, 1974), a procedure was devised for the large-scale isolation of purified plasma membranes from calf thymocytes which would provide the quantity of starting material required for this study. These plasma membranes contain at least eight glycoproteins which vary in molecular weight from 25 000 to 220 000, as revealed by periodic acid–Schiff reagent staining following electrophoresis of the membranes in sodium dodecyl sulfate–polyacrylamide gels. The initial plan of attack was to separate each of these glycoproteins, recognizing that there could well be more than eight. A partial separation of these glycoproteins was achieved by chromatography of sodium dodecyl sulfate solubilized membranes on hydroxylapatite columns followed by gel filtration on Bio-Gel P-300. The sugar composition and lectin-binding properties of glycopeptides derived from some of these partially purified glycoprotein fractions have been reported (Kornfeld, 1974). More recently, solubilization of the membranes in Triton X-100 followed by affinity chromatography on columns of lentil-lectin–Sepharose and *Ricinus communis* lectin–Sepharose revealed that the majority of the thymocyte membrane glycoproteins contain oligosaccharide chains with affinity for both lectins. The mixture of membrane glycoproteins which adsorbed to the lectin–Sepharose columns and could be eluted with methyl α-D-mannopyranoside and lactose, respectively, also had receptors for the erythro- and leuco-agglutinating lectins from red kidney beans and the lectin from the mushroom *Agaricus bisporus,* which have different sugar specificities than either the lentil or *Ricinus* lectins.

The similarity of lectin-binding properties of all the thymus membrane glycoproteins suggested that they might all contain a similar set of oligosaccharides. Therefore, a more efficient approach to determining the structure of the cell-surface oligosaccharides was to isolate these carbohydrate chains in the form of glycopeptides from the entire membrane pool of glycoproteins. Accordingly, in this study the glycopeptides isolated following delipidation and Pronase digestion of the purified plasma membranes have been fractionated and their carbohydrate structures analyzed.

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